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Effect of Food Restriction on Life Span and Immune Functions in Long-Lived Fischer-344 × Brown Norway F_1 Rats

G. FERNANDES,[1–3, 5] J. T. VENKATRAMAN,[3] A. TURTURRO,[4] V. G. ATTWOOD,[4] and R. W. HART[4]

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Life-long food restriction is known to slow aging and reduce the rate of occurrence of age-associated disease processes, but the mechanism by which this is accomplished is unknown. In this study we have examined the effect of food restriction on the proliferative response of spleen cells to mitogens and lymphokine production in 6-, 18-, and 30-month-old AL and FR Fischer-344 × Brown Norway (F-344×BNF$_1$) female rats whose average life span is 137 weeks on an ad libitum (AL) diet and 177 weeks on a food-restricted (FR) diet. In addition, the ability of food restriction to recall antigens was tested in 10-month-old rats by immunizing them with keyhole limpet and hen's egg albumin and measuring proliferative response of draining lymph node cells to these antigens. Our results indicated that the spleen-cell proliferative response to phytohemagglutinin and concanavalin A (Con A) was equal in 6- and 18-month-old rats but declined significantly in 30-month-old AL rats compared to FR rats. Although flow cytometric analyses did not reveal differences for CD4, CD8, and Ig$^+$ cells with age, a significant rise in memory T cells (Ox-22^{low}) in both CD4$^+$ and CD8$^+$ T-cell subset lineage was noted in AL-fed rats at 30 months of age. In FR rats, however, only a minimal shift of naive T cells (Ox-22^{high}) to memory cells was observed. In FR rats, the observed changes in the naive and memory T-cell subsets correlate well with the observed higher levels of the antiinflammatory interleukin-2 (IL-2) and lower levels of the proinflammatory cytokines such as IL-6 and tumor necrosis factor-α. The ability of food-restricted animals to recall antigens was lower compared to their age-matched controls, though the proliferative response to T-cell mitogen Con A and superantigen staphylococcal enterotoxin B was higher. These findings indicate that food restriction may selectively act to maintain a lower number of antigen-induced memory T cells with age, thereby maintaining the organism's ability to produce higher levels of IL-2 with age. In summary, the increased cell-mediated immune function noted in aged FR rats appears to be due to the presence of a higher number of naive T cells, which are known to produce elevated levels of the antiinflammatory cytokines, which may in part be responsible for reducing the observed age-related rise in disease.

KEY WORDS: Aging; cytokines; food restriction; immune functions; lymphocyte subsets.

INTRODUCTION

It is now well established that lifelong food restriction dramatically extends the life span and reduces the rate of occurrence of numerous physiological dysfunctions. Interestingly, calorie intake per unit of body weight remains constant for ad libitum-fed (AL) and food-restricted (FR) animals throughout their life span, indicating that there is no undernutrition or decrease in kilocalorie intake per gram weight between AL and FR animals (1, 2). Several possible mechanisms have been proposed for the role that moderate FR plays in disease prevention including the enhancement of the immune system, thereby leading to a decrease in the incidence of autoimmune diseases and extension of the life span (1–6). Immune senescence is characterized by a dysregulation of the immune system. At the biological level, the age-related changes of the complex network of interactions between the various participants of the immune system result in the loss of some activities and increases in other activities. At the clinical level, the disturbances of this interactive network lead to an inefficient and sometimes inappropriate or aberrant immune response. There is a decline in the production of naive lymphocytes by the central lymphoid organs, the thymus and bone marrow, leading to a reduced diversity and altered repertoire of antigen specificities recognized by the

[1] Department of Medicine, The University of Texas Health Science Center, San Antonio, Texas.
[2] Departments of Microbiology and Physiology, The University of Texas Health Science Center, San Antonio, Texas.
[3] Nutrition Program, State University of New York at Buffalo, Buffalo, New York.
[4] National Center for Toxicological Research, Jefferson, Arkansas.
[5] To whom correspondence should be addressed at Department of Medicine/Clinical Immunology, The University of Texas Health Science Center, 7703 Floyd Curl Drive, San Antonio, Texas 78284-7874.

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immune system. Thus, with age, there is a progressive decline in the capacity of the immune system to react with foreign antigens associated with an increased reactivity with autoantigens.

In the past several years, many age-related T-cell defects in immune responses have been documented in both humans and animals (7, 8). These defects have been linked to a number of disease events, including an increase in the susceptibility to malignancies, infections, and development of autoimmune diseases in the elderly. This decline in immune function may be due in part to a poor proliferative response of T and B cells to antigenic or mitogenic stimulation, whereas the immunological impairment appears to be due to a defect in cell cycle events and age-related alterations in T-cell subsets and their differentiation (7, 8). Aging is perceived as a process of impairment of immune functions; it is known that T cells from aged subjects have a reduced ability to produce interleukin-2 (IL-2). However, other functions seem to be upregulated in elderly subjects; indeed, IL-1, IL-3, IL-4, IL-6, interferon-α (IFN-α), and tumor necrosis factor-α (TNF-α) production are increased in both aged rats and aged humans. These cytokines are known to control B-cell differentiation, through isotype switch and Ig production.

Research carried out in the past two decades, in our laboratory and by other investigators, has indicated that FR may delay age-related impairment in the immune system and, particularly, of T-cell function. Such declines are generally believed to occur earlier than B-cell function (1, 5). Further, several recent advances in lymphocyte subset identification have made it possible to identify not only $CD4^+$ (helper) and $CD8^+$ (nonhelper) cells and their functions, but also new subsets within these two major T-cell subsets (8–10). The observed differences in cellular function among these subsets has also been reported to occur in both humans and rodents (11–13). Several changes in T-cell functions in humans and mice are now closely linked to changes within $CD4^+$ and $CD8^+$ T lymphocyte subsets based on the expression of various cell surface markers. Cells within the CD45 ($Pgp\text{-}1^+$) subset of T cells are increased in mice by about 50% with age and are found to be less responsive (i.e., refractory) to calcium activators as a function of age (14).

Shifts in the distribution of functionally distinct $CD4^+$ T-cell subsets, particularly increased accumulation of memory T cells, have been reported during aging in both mice and humans (15). Age-associated changes in cytokines, particularly the loss of IL-2 production, are implicated in the age-related impairment of T-cell proliferation, protein kinase C activation, and Ca^{2+} mobilization, which appear to be key events in the production of IL-2 and expression of IL-2 receptors and decline in the expression of specific mRNA with age (15–19). However, age-related changes in the production of other cytokines (such as IL-4, IL-6, and TNF-α) by memory T cells [in the absence of adequate regulatory levels of IL-2 and/or interferon-γ (IFN-γ)], may be proinflammatory and may reduce naive T-cell function.

Recently superantigens or bacterial toxins have also been implicated in the stimulation of T-cell subsets in both humans and rodents (20, 21). The superantigens, in combination with class II MHC molecules, stimulate $CD4^+$ $V\beta^+$ T cells by binding to a specific site in the variable portion of the β chain of the specific T-cell antigen receptor (22). It has also been reported that animals develop self-superantigens to eliminate autoreactive $V\beta$ T cells through clonal deletion (23). We have therefore also examined the effect of aging and food restriction on the ability of spleen cells to proliferate in response to both polyclonal mitogens and superantigens.

One of the concerns of all studies of aging, particularly aged rodents, is that the results obtained may reflect a disease state, rather than aging. F-344 rats, a popular model used in aging studies, have a high incidence of nephrotic disorders with age. The long-lived strain of F-344 × BNF1 rats have a median life span of 31 months and very few pathologies, even in their old age (24). The long-lived, inbred BN rat demonstrates an age-associated decrease in lymphoproliferation in response to concanavalin A (Con A); and these declines become apparent only after the age of median survival, 31 months (25). The present study was therefore designed to gain an understanding of the mechanisms through which FR may prevent or delay an age-associated decline in T cell-related immune functions and to gain further insight as to the role of FR in increasing life span in a long-lived rodent strain, such as F-344×BNF1 rats. In order to analyze these features, the proliferative response of spleen cells to mitogens and superantigens was used as a functional measure of the organisms. These studies are designed to establish whether both young AL and young FR rat cells proliferate to recall antigens with the same degree of intensity or behave differently from each other. In addition, the ability of FR young rats to recall antigens [keyhole limpet hemocyanin (KLH) or hen egg albumin (HEA) in Hunter's adjuvant] was also tested after immunizing the rats with these antigens and to determine the proliferative response of draining lymph node cells to these antigens including the response to Con A and superantigen staphylococcal enterotoxin B (SEB).

MATERIALS AND METHODS

Animals and Diets

Young and old Fischer-344×BNF$_1$ female rats were obtained from the FDA's National Center for Toxicological Research, Jefferson, Arkansas. The rats were raised in a specific pathogen-free environment at 23°C and were maintained on a light–dark cycle so that the lights were on from 0600 to 1800 as described by Duffy *et al.* (26). The rats were housed singly in polycarbonate cages with metal tops and hardwood chip bedding. The animals were fed the ad libitum diet (NIH) until 14 weeks of age (26). At 14 weeks of age, a 2-week ramping of dietary intake occurs, resulting in FR (60% of the mean caloric intake of the ad libitum group) by the end of the 16th week. FR was continued until the experiment was completed. Another group of rats was continued on the ad libitum diet. To carry out immunological analysis, the animals were first anesthetized and sacrificed by decapitation and spleens were aseptically removed for further analysis.

Spleen-Cell Preparation

Spleens were aseptically collected free of connective tissue, and single-cell suspensions were prepared. Spleens were minced gently in RPMI-1640 medium containing 5% heat-inactivated fetal bovine serum, 2 m*M* L-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin, sodium pyruvate, and 0.1 m*M* nonessential amino acids (27). The cell suspension was centrifuged at 100*g* and washed three times in the same medium.

Proliferative Response

Triplicate cultures were set up in wells of flat-bottom Corning microtiter plates, each containing 5×10^5 splenocytes in 0.2 ml of 1% fetal bovine serum (FBS) medium. The cells were cultured in the presence of phytohemagglutinin (PHA; 25 μg/ml; Wellcome, Beckenham, England), concanavalin A (Con A; 2.5 μg/ml; Pharmacia), and B-cell mitogens (BCM; 20 μg/ml). These mitogens have been tested in our laboratory, and concentrations giving optimal responses were selected. Superantigens (SEB; Toxin Technology, Sarasota, FL) were used at a concentration of 20 μg/ml based on our earlier experiments when we tested various concentrations of these superantigens. Time courses were conducted. After culturing for the indicated time, 0.5 μCi of [*methyl*-^{3}H]thymidine (6.7 Ci/mmol; New England Nuclear, Boston, MA) was added during the last 16 hr, and the incorporation of [*methyl*-^{3}H]thymidine was measured after harvesting the cells.

Flow Cytometric Analysis of Lymphoid Cell Subsets and IL-2 Receptors

Red cell-free spleen cells were stained with W3/25-PE (CD4), Ox8-PE (CD8), OX22-FITC (CD45RC), goat anti-rat Ig-FITC, and IL-2R-FITC (PharMingen, San Diego, CA, and Sera Labs). IL-2R level was measured in spleen cells cultured in the presence of Con A (5 μg/ml) for 48 hr. The cultured cells were passed through a Ficoll–Hypaque density gradient to remove dead cells and 1×10^6 cells were stained with anti-rat FITC-IL-2 receptor antibodies. All of the antibody-staining procedures were carried out in ice-cold phosphate-buffered saline (PBS)–3% fetal calf serum (FCS) (28). Antibodies were added to 1×10^6 cells in 100-μl portions at predetermined optimal staining concentrations, cell pellets were washed twice with 4 ml of PBS–3%FCS and resuspended in 200 μl of PBS–3% FCS, and the staining profile was determined using flow cytometry. Two-color contour plot analyses (FITC/PE) were carried out using standard techniques on a Becton–Dickenson FACStar Plus flow cytometer.

Interleukin-2 Production

IL-2 was assayed essentially in the same manner as described by Gillis *et al.* (29). IL-2 levels were measured in the cell-free supernatants collected from spleen cells cultured for 48 hr in the presence of Con A (2.5 μg/ml). IL-2 activity was measured using a murine IL-2-dependent cytotoxic T-cell line (CTLL-2). Approximately 5×10^4/ml CTLL cells in 100 μl were cultured with 100 μl of cell-free supernatant for 24 hr at 37°C in a humidified chamber. The cultures were harvested after pulsing with 0.5 μCi of [*methyl*-^{3}H]thymidine for 16 hr, and the activity was measured in a β-liquid scintillation counter. In calculating IL-2 activity, the lower limit of detection was arbitrarily defined as 3 SD above the mean of [^{3}H]thymidine incorporated by CTLL cells cultured in medium alone.

Interleukin-6

The proliferative effect of IL-6 was measured using a hybridoma cell line (B9 cells) as described (28). Prior to the assay, the cells were washed, resuspended at a concentration of 5×10^4 cells/ml, and 100 μl of cells was added to the wells in microtiter plates. Serially diluted cell-free supernatants from lipopolysaccharide

(LPS)-activated 24-hr cultured spleen cells were added, and the plates were incubated at 37°C in 5% CO_2 for 70 hr. The cells were pulsed with [^{3}H]thymidine for 4 hr and harvested. The [^{3}H]thymidine incorporation was measured in a β-liquid scintillation counter. The amount of IL-6 activity in the supernatants was determined from the rHuIL-6 standard curve.

Tumor Necrosis Factor

The presence of biologically active TNF in the LPS-activated spleen culture supernatants was measured as described (30). MTT assay was used to measure viability of cells, and color developed was read at 620 nm on a Dynatech-MR5000 ELISA reader. The amount of TNF activity in the supernatants was derived from the rHuTNF standard curve. Data were expressed as a mean of triplicate cultures and converted to units by comparison to standard TNF-α (Genzyme, Boston, MA).

Analysis of PGE_2

Prostaglandin E_2 (PGE_2) levels in cell-free supernatants of spleen cells cultured in the presence of lipopolysaccharides were analyzed by radioimmunoassay (31).

Measurement of Proliferative Responses to Recall Antigens

F-344×BN rats (10m old) were immunized in their foot pads with either 100 μg of HEA with Hunter's adjuvant or KLH-CFA (once), and the rats were sacrificed 2 weeks after immunization. A draining lymph node cell suspension was prepared in RPMI-1640 supplemented with 10 m*M* HEPES, 2 m*M* L-glutamine, 1 m*M* pyruvate, 50 m*M* 2-mercaptoethanol (2-ME) with 5% FBS, and 100 μg/ml DNase (Sigma, St. Louis, MO). The cells were cultured in the presence of mitogens (Con A, 0.5 μg/well), superantigen (SEB, 4 μg/well), and HEA (4 μg/well) or KLH (4 μg/well) for 72 hr for measurement of [^{3}H]TdR incorporation.

Statistical Analysis

The data were tested for statistical significance using Student's *t* test.

RESULTS

Survival Rate

The life span of Fischer-344×BNF_1 rats is significantly longer when the animals are fed a diet restricted by 40% of total calories compared to the AL diet (Fig. 1). The median life span of the ad libitum-fed rats was 138 ± 3.4 weeks, compared to the food-restricted animals, which had a median life span of 185 ± 3.4 weeks. The food-restricted animals had significantly lower body weights (Fig. 2). However, due to differences in body and organ weights, the food consumption per unit body mass was similar for AL and FR animals, indicating that FR does not cause malnutrition throughout the life span.

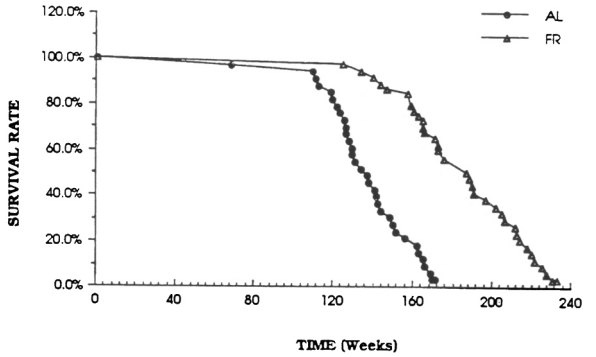

Fig. 1. Effect of food restriction on the survival rate of F-344×BN female rats (data from 34/group).

Proliferative Response to Mitogens and Superantigens

The proliferative response of spleen cells to mitogens (PHA, Con A, and BCM) was maximum at 48 hr following stimulation. The mitogenic effect on the proliferative response of spleen cells was mitogen dependent. For example, the response of spleen cells to PHA increased with age and was higher in the FR animals, while the response to Con A generally decreased with age but was improved by food restriction (Fig. 3). The response of spleen cells to BCM decreased with age, and the response was lower in the spleen cells of aging FR animals compared to AL-fed rats. Both aging and food restriction had a marked effect on proliferative response

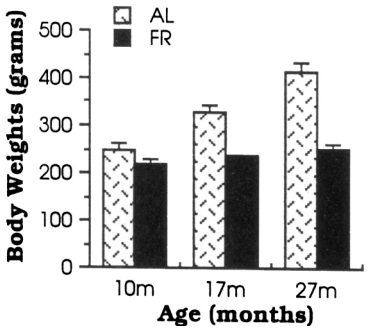

Fig. 2. Influence of food restriction on the body weights of F-344×BN female rats. Values are means ± SE of 34 rats per group.

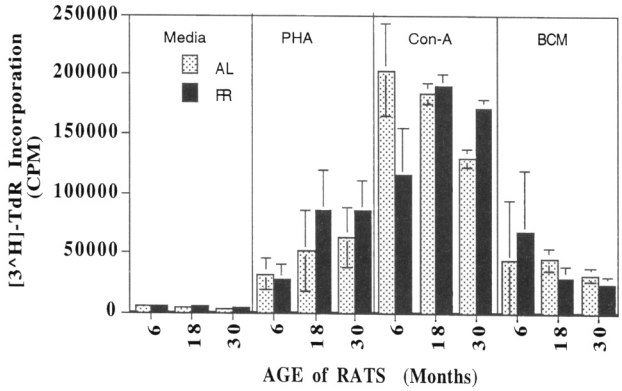

Fig. 3. *Influence of food restriction on proliferative responses of spleen* cells to mitogens in young and old F-344×BN rats. Values are means ± SE of five rats per group.

of spleen cells to superantigen (SEB). Aging significantly ($P < 0.01$) decreased the response of spleen cells to superantigens, while food restriction significantly delayed the decline in the proliferative response of spleen cells to superantigen exposure (Fig. 4), indicating the presence of higher naive T cells in FR-fed mice.

Lymphoid Cell Subsets

Phenotypic changes in young AL and FR rats is minimum in CD4, CD8, and Ig^+ cells (Table I). The FR rats had a slightly higher number of $CD4^+$ cells and lesser number of $CD8^+$ cells, though the values were not statistically significant compared to the AI groups. The percentage of Ig^+ cells declined with age in both AL and FR groups. The most striking effect was found in cells stained with Ox22 antibodies, where changes in the density (higher number of low-density population) were found in the 30 month-old AL-fed rats. Contour plots of

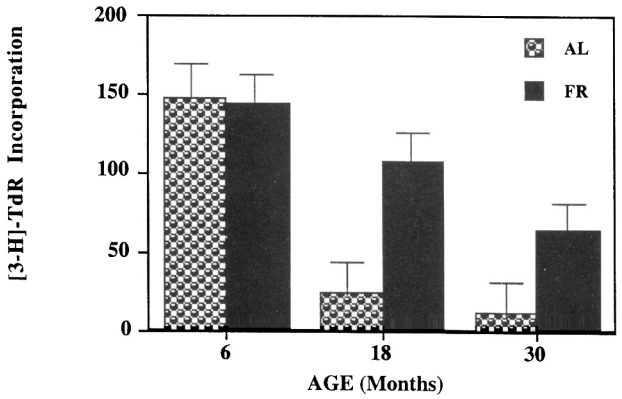

Fig. 4. Effect of food restriction on proliferative responses of spleen cells to SEB in young and old F-344×BN rats. Values are means of four rats per group.

Table I. Influence of Food Restriction on Lymphocyte Subsets in Aging F-344×BN Rats[a]

Age (mo)	Diet	$CD4^+$ (%)	$CD8^+$ (%)	Ig^+ (%)
6	AL	28.6 ± 6.8[b]	28.7 ± 4.3	39.6 ± 3.5
6	FR	33.0 ± 2.7	28.6 ± 5.1	42.7 ± 1.9
18	AL	26.4 ± 6.1	29.6 ± 0.6	34.0 ± 4.3
18	FR	36.8 ± 3.2	27.3 ± 4.7	38.2 ± 2.2
30	AL	29.0 ± 4.4	33.0 ± 4.2	33.9 ± 4.4
30	FR	31.5 ± 4.4	28.9 ± 2.8	32.9 ± 2.1*

[a] RBC-free spleen cells were stained with FITC-monoclonal antibodies and analyzed by flow cytometry.
[b] Values are means ± SE of four animals per group.
* Mean significantly different vs 6-month FR.

cells double-stained for CD4/Ox22 and CD8/Ox22 surface markers indicated no changes in density in young AL and FR rats (Fig. 5). However, in contrast, both $Ox22^+CD4^+$ and $Ox22^+CD8^+$ cell loss in the high-density population occurred in 30-month-old AL-fed rats. When the percentage distribution of cells stained for $Ox22^+$ and $Ox22^-$ and both CD4 and CD8 subsets was presented in the form of bar graphs, it was clear that $Ox22^+$ (naive) cells decreased significantly in AL-fed 30-month-old animals, while $Ox22^+$ cells were significantly higher in 30-month-old-FR animals in both CD4 and CD8 subsets (Fig. 6). Similarly, percentages of $Ox22^-$ (memory cells) were significantly higher in the 30-month-old AL-fed animals and food restriction significantly lowered the percentage of Ox22-cells in both CD4 and CD8 subsets. Thus, food restriction appears to prevent changes in the ratio of naive-to-memory cells in 30-month-old rats.

Interleukin-2 Receptors

The effect of aging and food restriction on the expression of IL-2 receptors in rat spleen cells cultured in the presence of Con A is presented in Fig. 7. A decrease in the number of IL-2 receptors occurred as the rats aged, but food restriction appears to slow this effect significantly.

Cytokine Production

The effect of food restriction and aging on cytokine production by spleen cells is presented in Table II. IL-2 production by cultured spleen cells decreased significantly with age ($P < 0.05$), and food restriction prevented this decline. Both IL-6 and TNF production by spleen cells cultured in the presence of LPS increased with age, but food restriction generally lowered the levels of these two cytokines in 30-month-old rats. PGE_2 production was significantly lower in 30-month FR rats

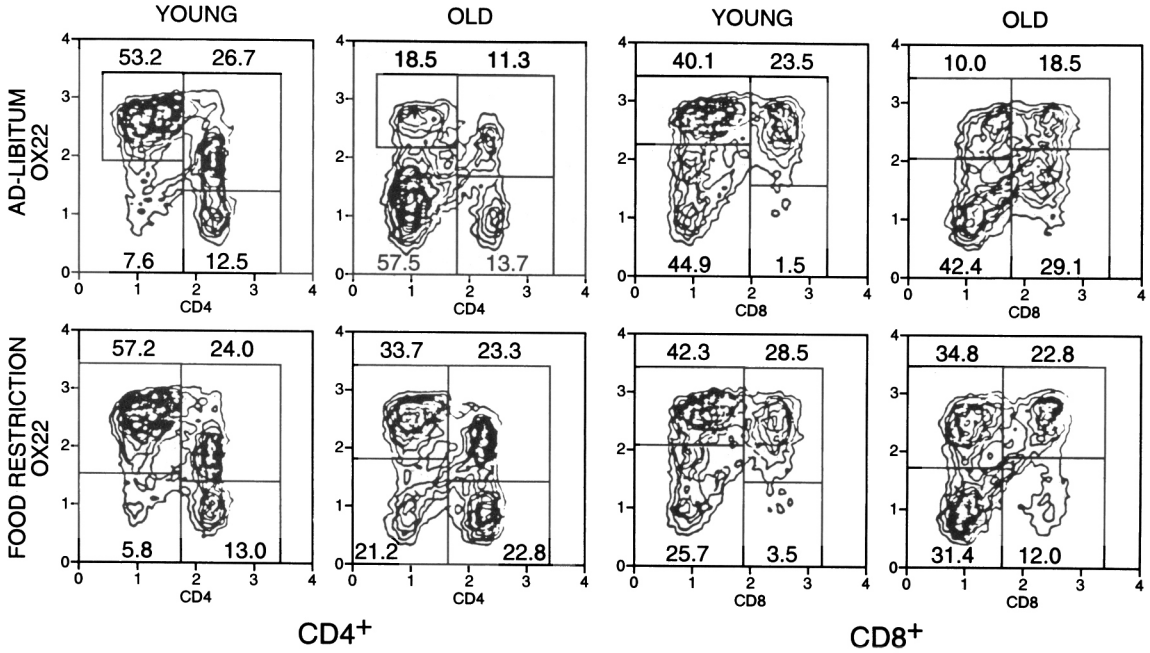

Fig. 5. Contour plots showing the effect of food restriction on the number of naive and memory cells in $CD4^+$ and $CD8^+$ subsets in the spleen cells (double stained with CD4 and Ox22 or CD8 and Ox22 antibodies) of young (6-month) and old (30-month) F-344×BN rats. Though the experiment was repeated in four or five animals per group, the contours presented here are for one animal per group.

compared to 30-month AL rats. Thus, the overall changes normally found to occur with age were slowed or delayed by food restriction.

Measurement of the Proliferative Response to Recall Antigens

The ability of FR rats to recall antigens appears to be lower than that of age-matched AL-fed animals, indicating a possible difference in naive and memory T cells (Fig. 8). Further, the proliferative response to Con A was also higher in FR animals. The response to HEA with Hunter's adjuvant was much higher than to KLH with CFA. The response to SEB (4 μg/well) was low in immunized rats, but still FR rats showed a higher response to SEB.

DISCUSSION

Several mechanisms have been proposed for understanding the protective role of FR in extending life span (1–6). Recently the decline of immunologic function has

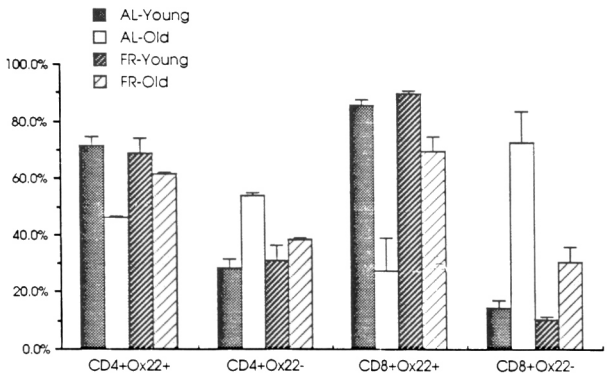

Fig. 6. Effect of food restriction on the proportion of naive and memory cells in young and old F-344×BN rats. Values are means ± SE of four rats per group.

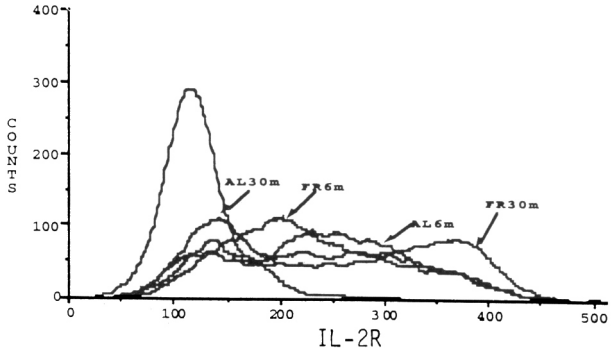

Fig. 7. Influence of food restriction on IL-2R level as analyzed by flow cytometry in 48-hr Con A-activated spleen cells of 6- and 30-month-old AL and FR F-344×BN rats.

Table II. Effect of Food Restriction on Cytokine Production by Spleen Cells of Young and Old F-344×BN Rats[a]

Cytokine	6 mo		30 mo	
	AL	FR	AL	FR
IL-2 (U/ml)	920 ± 180[b]	884 ± 185	475 ± 90*	724 ± 140
TNF (U/ml)	65 ± 13	51 ± 9	75 ± 15	65 ± 10
PGE_2 (pg/ml)	1,200 ± 200	1,040 ± 140	1,100 ± 180	630 ± 85**
IL-6 (U/ml)	13,200 ± 2,500	14,000 ± 4,010	22,000 ± 3,000	17,800 ± 3,100

[a] Spleen cells (5×10^6/ml) were cultured with either Con A for 48 hr or LPS for 24 hr and cytokines were analyzed in the cell-free supernatants. IL-2, IL-6, and TNF were quantitated by bioassay, and PGE_2 by radioimmunoassay.

[b] Values are means ± SE of observations from three or four animals.

* Mean IL-2 production significantly different from the mean of the 6-month AL group at $P = 0.05$.

** Mean PGE_2 production significantly different from the mean of the 6-month AL and 30-month AL groups at $P = 0.05$.

been linked to the functional loss of T-cell subsets with age, although the percentage of total B and T lymphocytes was less altered with age. Data from the present study revealed that food restriction may be preventing or delaying an age-associated decline in T cell-related immune functions by preventing a rise in memory T cells, thereby slowing the increase in the production of proinflammatory cytokines. Aged FR rats generally exhibited a better proliferative response of lymphocytes to mitogens and superantigens than aged AL-fed rats. In the past we also found that IL-2 receptor expression was higher in aged FR rats compared to their age-matched AL-fed controls (27, 32).

It has been shown that changes in T-cell functions in humans and mice are closely linked to changes within $CD4^+$ and $CD8^+$ T-lymphocyte subsets based on the expression of various cell surface markers. The increase in immature $CD2^+CD3^-$ T cells is an aging phenomenon related to declining T-cell proliferation (33). These lymphocyte subsets have in turn been associated with the secretion of distinct proinflammatory or antiinflammatory cytokines. Cells within the CD45 (Pgp-1^+) subset of T cells in mice are increased by about 50–75% with age and are less responsive to calcium activators (14). The impaired response to costimulation mediated by CD28

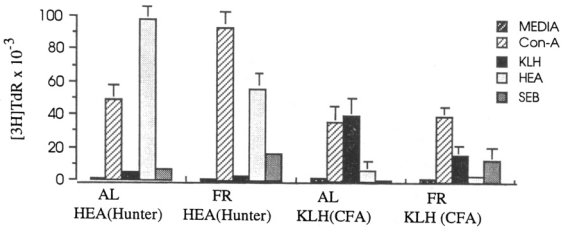

Fig. 8. Effect of food restriction in 10-month-old F-344 rats to recall antigens. Ten-month-old AL and FR rats were immunized with 100 μg of HEA with Hunter's adjuvant or KLH-CFA. The draining lymph nodes were collected from these animals 2 weeks later and were cultured with Con A, KLH, HEA, or SEB to determine the proliferative response.

on T cells from aged mice may be an important factor in the reduced T-cell responses associated with aging (34).

Goonawardene and Murasko (35) reported that food restriction does act to delay age-related changes in the proliferative response of spleen cells to Con A in BN rats. In addition, food restriction delays the plateau in Con A-induced IFN production seen after 23 months of age in AL F-344 × BNF1 rats (35). As mice age, spontaneous changes occur in the receptor repertoire of their T cells. The receptor repertoire of $CD4^+$ T cells does not change with age. In contrast, however, the percentage of $\alpha\beta^+$, $CD8^+$ T cells bearing particular Vβ elements varies considerably between individual aged mice, although it is remarkably consistent among individual young animals within a given strain. CD4/CD8 ratio drops steadily as a function of age. Shifts in CD4/CD8 ratio were not due to increased numbers of $CD8^+$ T cells in spleen and lymph nodes; rather $CD4^+$ T cells disappeared from aging mice more rapidly than $CD8^+$ T cells (36).

Recent studies have provided evidence that aging in humans is associated with a significant decrease in numbers of naive $CD45RA^+$ and $CD4^+$ T cells and an increase in numbers of $CD45RO^+$ CD29(hi) $CD4^+$ memory T cells in humans (37–40). The existence of a similar subset of T cells within either $CD4^+$ or $CD8^+$ T cells in young rats has also been reported (41, 42). The T cells that express high levels of Ox-22 (CD45R) also produce high levels of IL-2 in response to alloantigens and mitogens in young rats. Although both $Ox\text{-}22^+$ and $Ox\text{-}22^-$ T cells can provide help for B cells in primary antibody response, during *in vivo* activation the $Ox\text{-}22^+$ naive T cell converts into an $Ox\text{-}22^-$ memory T cell, which alone will be able to a support secondary B-cell response. Identification of T-cell subsets within $CD4^+$ and $CD8^+$ T cells in rats is relatively new and changes with age in naive to memory T cells in rats has not yet been reported. CD44 or PGP-1 is a transmembrane leukocyte adhesion-related glycoprotein which is often

expressed at a greater density on the membranes of memory T lymphocytes ($CD44^{hi}$) compared to naive T cells ($CD44^{lo}$). The proportion of PGP^{hi} or $CD44^{hi}$ cells among T cells is increased with advancing age. Our earlier studies on long-lived $B6D2F_1$ mice revealed that food restriction may be delaying aging in these mice by delaying age-related losses in naive cells, IL-2 production, and proliferative response of spleen cells to mitogens and superantigens (43). These results suggest that T-cell senescence with age is also associated with defective apoptosis, and the CD2-fas transgene allows maintenance of Fas apoptosis function and T-cell function in aged mice comparable to that of young mice (44).

Studies have shown that memory T cells can produce higher levels of IFN-γ compared to naive T cells (45, 46). It is now well established that aging can lower the production of IL-2, which is crucial for maintaining several T-cell functions. T cells in mice expressing high levels of $Pgp\text{-}1^{high}$ were found to generate fewer changes in Ca^{2+} compared to $Pgp\text{-}1^{low}$ naive T cells when exposed to either Con A, anti-CD-3 antibody, or low doses of ionomycin (14). Therefore, each subset may vary in its function due to the differences in its calcium extrusion pump. There is a dysregulation of cytokine production with an increased production of IL-4, IL-5, and IL-6 associated with a decreased production of IL-2, which may reflect an altered ratio of activity between the Th1- and the Th2-cell subsets. A similar dysregulation of cell-mediated immunity is manifested by an altered balance in cytokine production by T cells from old compared to young subjects (47). Thus, the production of IL-2, IL-3, and granulocyte macrophage-colony stimulating factor (GM-CSF) by T cells from old subjects is decreased, although the production of IL-4, IL-5, and IL-6 is undiminished or actually increased (47).

Aging is accompanied by dramatic shifts in the subset compositions of splenic $CD8^+$ cell pools, which contribute significantly to their increased capacity to produce IFN-γ at the population level (48). IL-3, IL-4, IL-5, IL-6, and IL-10 have been shown to have age-associated changes in AL-fed mice. IL-4 has been shown to increase with age in AL-fed mice in short-term cultures (49, 50). The long-lived, inbred BN rat demonstrates an age-associated decrease in lymphoproliferation in response to Con A; however, these declines become apparent only after the age of median survival, 31 months. Significant declines in IL-2 production after Con-A stimulation are reported to occur after median survival in BN rats (25). The imbalance in IL-2 and IFN production may reflect a dysregulation that results in a decreased proliferative response of lymphocytes with increasing age (25).

Changes in the proportion of cytokines, such as IL-4, IL-6, and TNF-β by memory T cells during aging in the absence of adequate regulatory levels of IL-2, may be proinflammatory and may reduce the naive T-cell function (35). However, food restriction does not delay the loss of age-related decline in Con A-induced IL-2 production (35).

However, experiments are required to confirm such possibilities by undertaking new *in vitro* studies with separated cells. Rat $CD4^+$ memory cells lack expression of the CD45RC or Ox22 surface antigen (41, 42), and it has been suggested that rat $CD4^+45RB^-$ cells exhibited a more vigorous response to polyclonal stimuli including IL-2 production, proliferation, IFN-γ, and TNF-β production. The capacity for IL-10 synthesis by the splenic $CD4^+$-cell pool is increased with age, and the age-related shift toward a predominance of $CD4^+CD44^{hi}$ cells in the peripheral tissues accounts for this quantitative change in IL-10 gene expression (51). The differential expression of CD45 isoforms has been suggested as a marker for stages of postthymic T-cell development, that is, $CD45RA^+CD45RO^-$ T cells and $CD45RA^-CD45RO^+$ T cells are supposed to be naive and memory cells, respectively. Recently, several adhesion molecules have been shown to be upregulated on the cell surface of memory T cells and have been suggested to serve as a memory marker. In $CD4^+$ T cells, the proportion of $LFA\text{-}1^{high}$ cells among $CD45RA^{high}CD45RO^-$ T cells remained low in all age groups and did not show significant accumulation with age. $CD4^+CD45RA^-CD45RO^{high}$ T cells expressed LFA-1 at a higher level than $CD4^+CD45RA^{high}CD45RO^-$ T cells (52).

Gradual age-associated shifts in the subset composition of the splenic $CD4^+$-cell pool in C57BL/6NNia mice underlie progressive changes in the patterns of cytokine gene expression by this cell group (51).

Results from the present studies also indicate that the proliferative response of spleen cells to superantigen (SEB) is decreased by aging, but food restriction prevents this loss. The binding of T cells to superantigen is very specific for certain Vβ regions which are linked to clonal deletion of self-reactive T cells. It will be of interest to compare the loss of Vβ repertoire with age in both AL and FR rats. The role of accessory T cells in the temporal expression of several key T-cell activation-associated antigens has been studied in healthy elderly. The response of PBMN cells to PHA was lower in the elderly. The number of T cells expressing CD71, CD25, CD38, or HLA-DR was lower in the elderly. T cells from healthy elderly show selective deficiencies in their capacity to respond to mitogenic stimuli and suggest that

impaired PHA responsiveness is due partly to defective accessory cell-derived signals (53). While total lymphocyte numbers altered only marginally in the aged (>70 years), there were significant changes in the distribution of various subpopulations; e.g., there were lower numbers of $CD3^+$ and $CD8^+$ cells and higher numbers of $CD16^+$(NK) cells. Shifts in the distribution of regulatory T-cell subsets may play a role in age-related changes in immune response (53). Signal transduction pathways involving CD2, except IL-7-mediated events, are reported to be essentially intact in old memory $CD4^+$ T cells, while on the other hand, several cofactors, namely, IL-2, IL-6, IL-7, and to a lesser extent, IL-1β and PMA, failed to support adequately CD2-induced activation in old $CD4^+CD45RA^+$ T cells, suggesting severe and multiple signaling deficiencies in this subset (54).

We have observed less decline in the intracellular calcium levels, fewer changes in fluidity, and lower levels of arachidonic acid (20:4n-6) in the phospholipid fractions in spleen cells of aging food-restricted Fischer-344 rats compared to the AL animals in the same age group (32, 55). Food restriction may be delaying the loss in age-related immune T-cell functions by acting on the lipid composition of lymphoid cells. This change in lipid composition may then assist in providing a favorable environment for optimal expression of IL-2 and Vβ-positive T-cell subsets as well as greater IL-2 production for optimum proliferative response of spleen cells.

In addition, our earlier data indicated that food restriction prevents the rise in immunosuppressive arachidonic acid (20:4n-6) and PGE_2 levels in spleen cells of Fischer-344 rats. We have observed an increase in 18:2 levels and a decrease in 20:4 levels in spleen cells of 19-month-old FR rats compared to the AL group of the same age (55). Recent reports strongly suggest that prostaglandins have a major inhibitory role in IL-2 receptor binding and/or IL-2 mRNA expression as well as decreasing the proliferative response of spleen cells (56). Food restriction may provide the appropriate physical characteristics or "microenvironment" in the membrane to facilitate influx of Ca^{2+} and induce optimum proliferative response by preserving membrane-bound receptor functions during aging (32, 55).

Naive and memory T cells may differ fundamentally in their activation requirements, and suggest that the accumulation, with age, of memory T cells accounts for the low responsiveness of old mice to noncognate mitogens such as Con A and SEB (57). The recall antigen experiments were designed to determine the response of AL and FR animals to recall antigens (KLH and HEA) when they were immunized with these antigens prior to determining the proliferative response to these antigens. Our results suggested that the ability of FR rats to recall antigens was lower, though the response to Con-A and superantigen SEB was higher in these animals. These observations may suggest a possible difference in the number in memory T cells. Better response of draining lymph node cells to Con A in the immunized FR animals is consistent with our data obtained for spleen cell proliferative response in nonimmunized animals. Better response to SEB by immunized FR animals may suggest an increased number of certain CD4 Vβ T cells, or the number of cells may be the same but they respond to SEB more efficiently compared to age-matched AL-fed animals. The recall antigen studies were carried out in 10-month-old AL and FR animals to test whether there was a differential response to recall anigens by FR animals. Currently experiments are in progress in our laboratory to test the ability to recall antigens in rats immunized at different ages to test the effect of food restriction on long-term memory.

It is clearly evident from the present studies that one of the mechanisms through which food restriction delays the age-associated decline in T cell-related immune functions may be through preventing a rise in memory cells and thereby preventing an increase in the production of proinflammatory cytokines. Further studies should be carried out in purified T cells, including $CD4^+$ and $CD8^+$ subsets, in order to explain some of the immunological variables that occur with age in both AL and FR rats. Finally, the present study also provides further evidence that moderate food restriction, which extends the life span through selectively delaying changes in T-cell subset function, may have relevance in delaying immunological dysfunction with age in humans by reducing the calorie intake.

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REFERENCES

1. Weindruch R, Walford R: *In* The Retardation of Aging and Disease by Dietary Restriction. Springfield, IL, Charles C Thomas, 1988
2. Masoro EJ: Retardation of the aging processes by food restriction: A search for mechanisms. ISI Atlas Sci Biochem 329–332, 1988

3. Yu BP, Masoro EJ, McMahan CA: Nutritional influences on aging of Fischer-344 rats: I. Physical, metabolic, and longevity characteristics. J Gerontol 40:657–670, 1985
4. Good RA, Gajjar AJ: Diet, immunity, and longevity. *In* ML Hutchinson, HN Munro (eds). New York, Academic Press, 1986, pp 235–249
5. Fernandes G: Nutritional factors: Modulating effects on immune function and aging. Pharmacol Rev 36:123S–129S, 1984
6. Harrison DE, Archer JR, Astle CM: Effects of food restriction on aging: Separation of food intake and adiposity. Proc Natl Acad Sci USA 81:1835–1838, 1984
7. Makinodan T, Chang MP, Norman DC, Li SC: Vulnerability of T cell lineage to aging. *In* Aging and Immune Response—Cellular and Humoral Aspects, E Goidl (ed). New York, Marcel Dekker, 1987, pp 27–44
8. Thoman ML, Weigle WO: The cellular and sub-cellular bases of immunosenscene. Adv Immunol 46:221–261, 1989
9. Bloom ET, Umehara H, Bleackley RC, Okumura K, Mostowski H, Babbitt JT: Age-related decrement in cytotoxic T lymphocyte (CTL) activity is associated with decreased levels of mRNA encoded by two CTL-associated serine esterase genes and the perforin gene in mice. Eur J Immunol 20:2309–2316, 1990
10. Vitetta ES, Berton MT, Burger C, Kepron M, Lee WT, Yin X-M: Memory B and T cells. Annu Rev Immunol 9:193–217, 1991
11. Miller RA: Defective calcium signal generation in a T cell subset that accumulates in old mice. Ann NY Acad Sci 568:271–276, 1989
12. Weksler ME, Russo C, Siskind GW: Peripheral T cells select the B cell repertoire in old mice. Immunol Rev 110:173–185, 1989
13. Murasko DM, Weiner P, Kaye D: Decline in mitogen induced proliferation of lymphocytes with increasing age. Clin Exp Immunol 70:440–448, 1987
14. Lerner A, Yamada T, Miller RA: PGP-1^{hugh} lymphocytes accumulate with age in mice and respond poorly to concanavalin-A. Eur J Immunol 19:977–982, 1989
15. Grossmann A, Ledbetter JA, Rabinovitch PS: Aging-related deficiency in intracellular calcium response to anti-CD3 or concanavalin-A in murine T cell subsets. J Gerontol 45:B81–B86, 1990
16. Holbrook NJ, Chopra RK, McCoy MT, Nagel JE, Powers DC, Alder WH, Schneider EL: Expression of IL-2 and IL-2R in aging rats. Cell Immunol 120:1–9, 1989
17. Wu W, Pahlavani M, Cheung HT, Richardson A: The effect of aging on the expression of interleukin-2 messenger ribonucleic acid. Cell Immunol 100:224–231, 1986
18. Proust JJ, Kittur DS, Buchholz MA, Nordin AA: Restricted expression of mitogen-induced high-affinity IL-2 receptors in aging mice. J Immunol 141:4209–4216, 1988
19. Fong TC, Makinodan T: In situ hybridization analysis of the age-associated decline in IL-2 mRNA expressing murine T cells. Cell Immunol 118:199–207, 1989
20. Janeway CA Jr: Self superantigens? Cell 63:653–661, 1990
21. Callahan JE, Herman A, Kappler JW, Marrack P: Stimulation of B10.BR T cells with superantigenic staphylococcal toxins. J Immunol 144:2473–2479, 1990
22. Coffin JM: Superantigens and endogenous retroviruses: A confluence of puzzles. Science 255:411–413, 1992
23. Marrack P, Kappler J: The staphylococcal enterotoxins and their relatives. Science 248:705–711, 1990
24. Bronson RT: Rate of occurrence of lesions in 20 inbred and hybrid genotyoes of rats and mice sacrificed at six month intervals during the first year of life. *In* Genetic Effects on Aging II, DE Harrison (ed). Caldwell, NJ, Telford Press, 1992, pp 280–358
25. Goonewardene MI, Murasko DM: Age associated changes in mitogen induced proliferation and cytokine production by lymphocytes of the long-lived Brown Norway rat. Mech Age Dev 71:199–212, 1993
26. Duffy PH, Feuers RJ, Leaky JEA, Nakamura KD, Turturro A, Hart RW: Effect of chronic calorie restriction on physiological variables related to energy metabolism in male Fischer-344 rat. Mech Age Dev 48:117–133, 1989
27. Iwai H, Fernandes G: Immunological functions in food-restricted rats; Enhanced expression of high-affinity IL-2 receptors on splenic T cells. Immunol Lett 23:125–132, 1989
28. Sharrow SO: Analysis of flow cytometry data. *In* Current Protocols in Immunology, Vol 1, JE Coligan, AM Kruisbeek, DH Margulies, EM Shevach, W Strober (eds). New York, Green, Wiley–Interscience, 1991, p 52
29. Gillis SM, Ferm M, Ou W, Smith K: T cell growth factor: Parameters of production and a quantitative microassay for activity. J Immunol 120:2027–2031, 1978
30. Espevik T: A highly sensitive cell line, WEHI 164 clone 13, for measuring cytotoxic factor/tumor necrosis factor from human monocytes. J Immunol Methods 95:99–105, 1986
31. Flescher F, Fossum D, Gray PJ, Fernandes G, Harper M, Talal N: Aspirin-like drugs prime human T cells: Modulation of intracellular calcium concentrations. J Immunol 146:2553–2559, 1991
32. Fernandes G, Flescher E, Venkatraman JT: Modulation of cellular immunity, fatty acid composition, fluidity and $Ca2^{+}$ influx by food restriction in aging rats. Aging Immunol Infect Dis 2:117–125, 1990
33. Lesourd BM, Meaume S: Cell-mediated immunity changes in ageing, relative importance of subpopulation switches and of nutritional factors. Immunol Lett 40:235–242, 1994
34. Engwerda CR, Handwerger BS, Fox BS: Aged T cells are hyporesponsive to costimulation mediated by CD28. J Immunol 152:3740–3747, 1994
35. Goonewardene MI, Murasko DM: Age-associated changes in mitogen-induced lymphoproliferation and lymphokine production in the long-lived brown-Norway rat: effect of calorie restriction. Mech Age Dev 83:103–116, 1995
36. Callahan JE, Kappler JW, Marrack P: Unexpected expansion of CD8-bearing cells in old mice. J Immunol 151:6657–6669, 1993
37. De Paioli P, Battistin S, Santini GF: Age-related changes in human lymphocyte subsets: Progressive reduction of the CD4 CD45R (suppressor inducer) population. Clin Immunol 48:290–299, 1988
38. Sanders ME, Makgoba MW: Human naive and memory T cells: Reinterpretation of helper-inducer and suppressor-inducer subsets. Immunol Today 9:195–199, 1988
39. Walker C, Gauchat JF, De Weck AL, Stadler BM: Analysis of leucocyte markers in elderly individuals. Aging Immunol Infect Dis 2:31–37, 1990
40. Warren RP, Yonk LJ, Burger RA, Singh VK: Age-related changes in $CD45R^{+}$ and $CDW29^{+}$ helper T cells in human subjects. Aging Immunol Infect Dis 2:91–94, 1990
41. Ericsson PO, Lindén O, Dohlsten M, Sjögern HO, Hedlund G: Functions of rat $CD4^{+}$ T cell subsets defined by CD45RB: CD45RB cells have a much stronger response to recall antigens, whereas polyclonally activated cells of both subsets are equally efficient producers of IFN in the presence of exogenous IL-2. Cell Immunol 132:391–399, 1991
42. Powrie FM, Mason D: The MRC Ox22 $CD4^{+}$ T cells that help B cells in secondary immune responses derive from naive precursors with the MRC $Ox22^{+}CD4^{+}$ phenotype. J Exp Med 169:653–662, 1989

43. Venkatraman JT, Attwood VG, Turturro A, Hart RW, Fernandes G: Maintenance of virgin T cells and immune functions by food restriction during aging in long-lived $B6D2F_1$ female mice. Aging Immunol Infect Dis 5:13–25, 1994
44. Zhao T, Edwards CK, Mountz JD: Prevention of age-related T cell apoptosis defect in CD2-fas-transgenic mice. J Exp Med 182:129–137, 1995
45. Hayakawa K, Hardy RR: Phenotypic and functional alteration of $CD4^+$ T cells after antigenic stimulation. Resolution of two populations of memory T cells that both secrete IL-4. J Exp Med 169:2245–2250, 1989
46. Bottomly KA, Luqman M, Greenbaum L, Carding S, West J, Pasqualini T, Murphy DB: A monoclonal antibody to murine CD45R distinguishes CD4 T cell populations that produce different cytokines. Eur J Immunol 19:617–623, 1989
47. Weksler ME, Schwab R: The immunogenetics of immune senescence. Exp Clin Immunogenet 9:182–187, 1992
48. Ernst DN, Weigle WO, Noonan DJ, McQuitty DN, Hobbs MV: The age-associated increase in IFN-γ synthesis by mouse $CD8^+$ T cells with changes in frequencies of cell subsets defined by membrane CD44, CD45RB, 3G11, and MEL-14 expression. J Immunol 151:578–587, 1993
49. Kubo M, Cinader B: Polymorphism of age-related changes in interleukin production: differential changes of T helper populations, synthesizing IL-2, IL-3, and IL-4. Eur J Immunol 20:1289–1296, 1990
50. Hobbs MV, Weigle WO, Noonan DJ, Torbett BE, McEvilly RJ, Koch RJ, Cardenas GJ, Ernst DN: Patterns of cytokine gene expression by CD4+ T cells from young and old mice. J Immunol 150:3602–3614, 1993
51. Hobbs MV, Weigle WO, Ernst DN: Interleukin-10 production by splenic $CD4^+$ cell subsets from young and old mice. Cell Immunol 154:264–272, 1994
52. Okamura M, Fujii Y, Takauchi Y, Inada K, Nakahara K, Matsuda H: Age-related accumulation of LFA-1high cells in a CD8+CD45 RA high T cell population. Eur J Immunol 23:1057–1063, 1993
53. Xu X, Beckman I, Dimopoulos K, Ahern M, Bradley J: Age-related changes in the expression of T cell activation antigens following phytohemagglutinin stimulation. Exp Clin Immunogenet 9:203–211, 1992
54. Beckman I, Shepherd K, Firgaire F, Ahern M: Age-related defects in CD2 receptor-induced activation in human T-cell subsets. Immunology 86:533–536, 1995
55. Venkatraman JT, Fernandes G: Modulation of age-related alterations in membrane composition and receptor associated immune functions by food restriction. Mech Age Dev 63:27–44, 1991
56. Goodwin JS, Ceuppens J: Regulation of immune responses by prostaglandins. J Clin Immunol 3:295–315, 1983
57. Flurkey K, Stadecker M, Miller RA: Memory T lymphocyte hyporesponsiveness to non-cognate stimuli: A key factor in age-related immunodeficiency. Eur J Immunol 22:931–935, 1992